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Covalent lectin inhibition and application in bacterial biofilm imaging

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Abstract

Biofilm formation by pathogenic bacteria is a hallmark of chronic infections. In many cases, lectins play key roles in establishing biofilms. The pathogen Pseudomonas aeruginosa often exhibiting various drug resistances employs its lectins LecA and LecB as virulence factors and biofilm building blocks. Therefore, inhibition of the function of these proteins is thought to have potential in developing ‘pathoblockers’ preventing biofilm formation and virulence. Here, we describe for the first time a covalent lectin inhibitor specific to a carbohydrate binding site. In addition we report its application in the LecA-specific in vitro imaging of biofilms formed by P. aeruginosa.

Lectins are carbohydrate-binding proteins with very diverse functions that are found in all domains of life. These proteins play crucial roles in numerous processes such as cell-cell recognition, infection processes and immune defense. They are generally characterized by an intermediate to low affinity towards their carbohydrate ligands that is often overcome by Nature through multivalency of both the lectin receptors and their carbohydrate ligands resulting in avidity with an increase in apparent affinity.

Because these carbohydrate-binding proteins play essential roles in a number of pathological processes, they have become attractive targets for therapy. However, the fact that lectins display moderate affinities to their ligands renders this class of proteins as difficult targets for drugs. Despite this drawback, a number of recent success stories impressively demonstrated their potential for therapy: the selectin antagonist GMI-1070 is currently in phase III clinical trials and various FimH inhibitors are in the late preclinical stage.

Lectins are involved in infections with the Gram-negative bacterium P. aeruginosa, one important member of the often highly drug resistant ESKAPE pathogens which are Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa, and Enterobacter species and currently cause most of the severe hospital infections in western countries. The two bacterial lectins, LecA and LecB, are virulence factors and important for bacterial adhesion and biofilm formation. The latter is especially problematic as resistance against antibiotics inside a biofilm is increased by a factor of 10-1000. Thus, the inhibition of these lectins provides a promising way to dismantle the bacterium from the protective biofilm environment and restore immune defense and activity of antibiotics. Current approaches to inhibit both lectins range from small molecules to multivalent structures and are summarized in recent
reviews. We focus on the development of small molecules and recently published various potent glycomimetic inhibitors for the high affinity lectin LecB as inhibitors of \textit{P. aeruginosa} adhesion. In contrast, LecA only has an intermediate affinity for its monovalent D-galactose-derived ligands in the 50-100 µM range. Phenyl β-D-galactosides and derivatives showed an increased affinity of approx. 10 µM, \textit{e.g.} compound 1 (Figure 1), but despite a high number of derivatives analyzed, no further significant increase in potency could be achieved.

Covalent inhibition is one strategy to avoid dissociation of the inhibitor from the target and thus to persistently inactivate proteins. To date, a specific covalent inhibition of the carbohydrate binding site in a lectin has not been achieved despite attempts using squaric acid to target FimH or photoactivatable substituents for the targeting of galectins. The latter probes covalently bind to unspecific residues of the protein in proximity (3 Å) to the photoactivated center. The crystal structure of LecA reveals the presence of one cysteine residue (Cys62) in the carbohydrate binding domain (Figure 1). The specific targeting of cysteine residues with electrophilic warheads is a general strategy in the search for cysteine protease inhibitors, but has never been addressed in carbohydrate recognition domains. In order to target Cys62, we designed the two diastereoisomeric galactose-derived epoxides 2 and 3 (Figure 1) as potential covalent active site inhibitors of LecA.

\textbf{Figure 1:} A. Galactoside recognition by the bacterial lectin LecA (pdb: 3ZYF); B. Electrophilic epoxide derivatives 2 and 3 for the targeting of Cys62. The distances from Cys62-S to C6 and O6 of 1 are between 4.1 and 4.3 Å. (sc = side chain, bb = back bone)
Scheme 1: Synthesis of LecA-directed epoxides and competitive binding to LecA. (i) acetone, ZnCl₂, H₂SO₄; (ii) (COCl)₂, DMSO, NEt₃, CH₂Cl₂, -78 °C to 0 °C; (iii) PPh₃*MeI, NaH, DMSO; (iv) 70% HOAc aq.; (v) Ac₂O, pyridine; (vi) PhOH, BF₃*Et₂O, CH₂Cl₂, -20 °C - r.t.; (vii) NaOMe, MeOH, r.t.; (viii) mCPBA, NaHCO₃, CH₂Cl₂/MeOH;

The 8 step synthesis of epoxides 2 and 3 started from D-galactose (4) with epoxidation as the last step (Scheme 1). 4 was protected as diacetonide (→5) and the free primary hydroxyl group was oxidized under Swern conditions (→6) in good yields. Then, heptose 7 was established in a Wittig reaction followed by a change in protecting groups from acetonides to acetates (→8). Lewis acid catalyzed glycosylation of phenol with 8 gave phenyl β-glycoside 9 in good yield. 9 was deprotected in a Zemplén transesterification reaction to give olefin 10 in 97% yield. Late stage oxidation with mCPBA yielded the two diastereoisomeric epoxides 2 and 3 in 9% and 19%, respectively. The stereochemistry of 3 was established by X-ray crystallography in complex with LecA (see Figure 3).

Both epoxides 2 and 3 and olefin 10 were then tested in a recently developed competitive binding assay[12d] for inhibition of LecA. No inhibition was observed for olefin 10 up to 3 mM. In contrast, the epoxides showed a strong diastereoselectivity for inhibition of LecA: 3 was a good inhibitor with an IC₅₀ = 64 µM, whereas its diastereomer 2 was not recognized (IC₅₀ > 3 mM), indicating a specific binding of epoxide 3 to LecA. Covalent inhibitors usually show a time dependent reduction of IC₅₀s due to the accumulation at the protein. We have therefore studied the time dependency of the binding of 2, 3 and phenyl β-D-galactoside to LecA (Figure S21). The IC₅₀ of the latter non-
A covalent inhibitor stayed constant over time, inactive diastereomer 2 remained inactive, whereas the binding epoxide diastereomer 3 showed a time dependent decrease of the IC\textsubscript{50} values, indicative for covalent binding.

To assess the binding mode of 3 with LecA, we analyzed LecA in presence and absence of 3 by mass spectrometry (Figure 2). LC-MS measurements on intact protein level showed a mass shift of 268.1 Da when incubated with 3 and thus prove a covalent binding of the epoxide to LecA (Figure 2A, B). Attempts to enzymatically or chemically digest the LecA complex failed due to the extraordinary stability of LecA. In order to localize the binding site of the epoxide, an MS based sequencing using MALDI in source decay (ISD) was therefore performed for both samples (Figure 2C). MALDI-ISD experiments with c-ion series annotated in a range from 5000 to 8000 m/z were instrumental to identify the peptide sequence ranging from Arg48 to Thr74 and Asn71, respectively. For the LecA sample co-incubated with 3, a mass increase of 268 Da to the c62 ion indicated the binding of compound 3 to Cys62 of LecA, with some unspecific binding to Cys57 also detected. Based on the mass increase of 268 Da to the c62 ion, 3 showed covalent binding to LecA and the nucleophile for the epoxide ring opening was the sulphydryl group of Cys62 in the carbohydrate recognition domain.

Figure 2: Covalent binding mode of 3 with LecA established by mass spectrometry. Deconvoluted intact protein MS spectra of LecA: A. without inhibitor, and B. with inhibitor 3; C. MALDI-ISD experiments with c-ion series annotated in a range from 5000 to 8000 m/z.
We then crystallized LecA in complex with epoxide 3 and solved the structure by X-ray crystallography (Figure 3). In this complex, 3 adopts a coordination to the calcium ion bound to LecA as it had been reported for other galactosides before.\textsuperscript{[11a,15]} Surprisingly, despite its orientation towards Cys62 the epoxide moiety in 3 is still intact and the covalent adduct could not be observed in this structure. These differences to the covalent adduct detected by mass spectrometry are likely a result of the different pH values of the buffers employed: lectin binding assays and mass spectrometry were performed at a physiologically buffered pH (7.4), whereas LecA was crystallized at pH 4.6. Numerous attempts to obtain LecA crystals with 3 as a covalent adduct by cocrystallization or soaking at neutral pH have been unsuccessful to date. All data collection of protein crystals incubated with diastereomeric epoxide 2 led to empty binding sites confirming the low affinity of 2 for LecA.

Figure 3: Crystal structure of epoxide 3 in complex with LecA at 1.80 Å resolution in the non-covalent binding mode obtained at pH 4.6 (pdb code 5MIH). A. Electron density displayed at 1σ for ligand and Cys62 side chain. B. Interaction of the ligand with LecA: the epoxy-oxygen atom accepts hydrogen bonds from His50 and one protein-bound water molecule. In addition, His50 established a CH-π interaction with the phenyl aglycon. In the crystal, the sulfur atom of Cys62 is 3.3 Å away from C7 of ligand 3.
In order to exploit this unique covalent lectin ligand for biological applications such as lectin specific staining, we synthesized alkyne-bearing derivatives that were then coupled to a fluorescent azide in a Huisgen dipolar cycloaddition (Scheme 2). Glycosyl donor 8 was reacted under Lewis acid catalysis with the acceptor monopropargyl hydroquinone to give the glycoside 11. Here, we first oxidized the peracetylated olefin 11 using mCPBA and the two diastereomeric epoxides 12 and 13 were obtained after chromatographic separation in 21% and 46% yield, respectively. Subsequently, the acetates were removed in a Zemplén type reaction to individually give the 14 or 15. Both were then tested for inhibition of LecA showing a comparable diastereoselectivity as observed for the unsubstituted phenyl derivatives 2 and 3 before: 6D epoxide 15 inhibited LecA with an IC$_{50}$ of 109 µM, whereas the diastereomeric 6L epoxide 14 was inactive (IC$_{50}$ > 3 mM). The stereochemistry of 14 and 15 was unambiguously assigned by combining the activity data and NMR chemical shift and coupling constant analysis and comparison with analogs 2 and 3. The active diasteromer 15 was then coupled in a copper(II)-catalyzed click reaction to the azide 16 to give fluorescent probe 17 in good yields.

Scheme 2: Synthesis of LecA-directed propargylated epoxides with LecA inhibition data and synthesis of fluorescent derivative 17. (i) hydroquinone monopropargyl ether, BF$_3$*Et$_2$O, CH$_2$Cl$_2$, -20 °C - r.t.; (ii) mCPBA, NaHCO$_3$, CH$_2$Cl$_2$, 0 °C - r.t.; (iii) NaOMe, MeOH, 0 °C; (iv) CuSO$_4$, sodium ascorbate, H$_2$O, DMF, r.t.;

The covalent nature of the binding of fluorescein-derivative 17 was then further studied by incubation with LecA. Complexes of LecA with 17 or with a non-covalent analog 18 were preformed as observed by high fluorescence polarization. In contrast to the covalent complex of 17 with LecA, 18 could be completely displaced from LecA with the competitive inhibitor methyl galactoside (Figure S22). Furthermore, the complex of LecA with 17 was analyzed by polyacrylamide gel electrophoresis under denaturing conditions (Figure 4). LecA incubated with 17
gave a single fluorescent band that was also stained by Coomassie corresponding to the molecular weight of the denatured monomer of LecA and thus yields further evidence for covalent binding.

Figure 4: Covalent binding of 17 to LecA established by SDS-PAGE. (A) Fluorescence imaging; (B) Coomassie staining; M* BenchMark fluorescent protein standard (Thermo), M# protein marker III (Applichem).

Tools to visualize the presence of carbapenem-resistant bacterial pathogens in a test tube by specific activity-based probes have recently been reported. The visualization of bacterial biofilm structures is of outstanding current interest, both, in vitro and in vivo. LecA is involved in the biofilm formation of P. aeruginosa and LecA-deficient strains were shown to have thinner biofilms with a reduced biomass. Because the expression of LecA is upregulated in biofilms and it is located extracellularly this protein is a promising target for the imaging of biofilms. We therefore explored whether the LecA-directed epoxides reported here can specifically stain biofilms of P. aeruginosa. Bacterial biofilms were grown using mCherry-expressing PAO1 wildtype bacteria and the corresponding LecA-deficient strain PAO1 ΔlecA and then analyzed by confocal fluorescence microscopy (Figures 5, 6). Under shaking growth conditions, bacterial aggregates of the biofilm were observed in the PAO1 wildtype strain, whereas the ΔlecA strain generally showed a heavily reduced number of aggregates with smaller sizes and therefore also a higher number of planktonic bacteria since bacterial growth is comparable (Figures 5, 6, S23). After addition of the LecA-directed dye 17 to the bacterial cultures, a specific staining of the wildtype biofilm aggregates was detected and no staining was visible in case of the aggregates formed by the ΔlecA strain. The green fluorescence originating from 17 was observed on the entire structure of the wildtype bacterial aggregates, whereas no or only a very faint color on the surface of the ΔlecA strain aggregates was observed without any detectable fluorophore inside these aggregates. The largest aggregate found for the ΔlecA strain was also analyzed (Figures S24-S26) and staining was LecA-specific and independent of aggregate size. Thus, the bacterial lectin LecA can be exploited as a target to visualize biofilms of P. aeruginosa using conjugates of LecA-ligands, such as the galactose-derived epoxide 17.
**Figure 5**: Galleries of LecA-dependent staining of *P. aeruginosa* biofilms with 17. *P. aeruginosa* PAO1 wt (A) or the lecA knockout (∆lecA) mutant (B), both expressing mCherry from pMP7605, were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with the covalent LecA ligand fused to fluorescein (17) for 10 - 30 min. Z-stacks (232 x 232 µm) were recorded every 2 µm at 561 nm for mCherry (red, A and B, upper panels) and 488 nm for fluorescein (green, A and B middle panels). The galleries show every 4th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm).

**Figure 6**: Three-dimensional imaging of LecA-dependent staining of *P. aeruginosa* PAO1 biofilms with 17. *P. aeruginosa* PAO1 wt (A) or the lecA knockout (∆lecA) mutant (B) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with 17 for 10 - 30 min. Z-stacks (232 x 232 µm) were recorded every 2 µm at 561 nm for mCherry (red) and 488 nm for fluorescein (green). The 3D images show merged images of both channels (488 nm and 561 nm) from top and side views.

In summary, we have developed the first covalent inhibitor of carbohydrate binding sites by rational structure-based design. Both diastereomers of the epoxygalactoheptoside 2 and 3 were synthesized and biologically evaluated. LecA displayed a strong diastereoselectivity for the 6D epimer 3 over its...
6L isomer 2. The binding site and its covalent nature at physiological pH was established using mass spectrometry-based sequencing and the non-covalent crystal structure of 3 in complex with LecA was solved at pH 4.6. Finally, we used the fluoresceine-derivative 17 for the LecA-specific staining of *P. aeruginosa* biofilms. Such conjugates may lead to the development of pathogen-specific imaging agents to localize bacterial biofilm-associated infections inside an infected host enabling pathogen- and tissue-directed therapy.

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**Author contributions**

S.W. performed and analyzed microbiology and biofilm experiments, D.H. performed all chemical syntheses except for compound 17 which was synthesized by R.S., I.J. and R.S. performed the competitive LecA inhibition and binding assays, M.H. and R.M. performed and analyzed protein MS and small molecule HRMS experiments, A.V. and A.I. established and analyzed the crystal structure of 3 in complex with LecA, A.T. conceived and designed the study, A.T. wrote the paper with input from all authors.
Supporting Information

Electronic supporting information contain experimental details, $^1$H and $^{13}$C-NMR spectra, and LC-MS traces of 16 and 17. Crystallographic data of the complex of LecA with 3 are given in Table S1; Binding kinetics experiments and inhibitor displacement experiments, biofilm fluorescence microscopy data prior to staining, stained unprocessed raw data and processed data of the largest biofilm aggregate of the ΔlecA strain are given.

References


Supporting Information to

Covalent lectin inhibition and its application in bacterial biofilm imaging

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\textsuperscript{2} Deutsches Zentrum für Infektionsforschung (DZIF), Standort Hannover–Braunschweig (Germany)

\textsuperscript{3} Microbial Natural Substances, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), D-66123 Saarbrücken, Germany

\textsuperscript{4} Université Grenoble Alpes, CERMAV, F-38000 Grenoble, France; CNRS, CERMAV, F-38000 Grenoble, France.

\textsuperscript{*} corresponding author, email: alexander.titz@helmholtz-hzi.de
Figure S1: 1H NMR of 9 (DH142-5_Fr2)

Figure S2: 13C NMR of 9 (DH142-5_Fr2)
Figure S3: 1H NMR of 10 (DH159)

Figure S4: 13C NMR of 10 (DH159)
Figure S5: 1H NMR of 2 (DH174fr1)

Figure S6: 13C NMR of 2 (DH174fr1)
Figure S7: 1H NMR of 3 (DH174fr2)

Figure S8: 13C NMR of 3 (DH174fr2)
Figure S9: 1H NMR of 11 (DH247fr2)

Figure S10: 13C NMR of 11 (DH247fr2)
**Figure S11**: $^1$H NMR of 13 (DH249fr1)

**Figure S12**: $^{13}$C NMR of 13 (DH249fr1)
Figure S13: 1H NMR of 12 (DH249fr3)

Figure S14: 13C NMR of 12 (DH249fr3)
Figure S17: 1H NMR of 15 (DH252A)

Figure S18: 13C NMR of 15 (DH252A)
Figure S19: LCMS of FITC-Azide 13

Figure S20: LCMS of FITC labelled epoxide 6D-17
Figure S21: Kinetics of LecA inhibition of the two epoxide diasteromers 2 and 3 and the non-covalent inhibitor phenyl β-D-galactoside at r.t. (A) and 37 °C (B), respectively.

Figure S22: Displacement of 17 (A) or 18 (B) from their complexes with LecA using the competitive inhibitor methyl α-D-galactoside as studied by fluorescence polarization.
Table S1: Data collection and refinement statistics for LecA structure with 3

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*Values in parentheses are for highest-resolution shell.
Figure S23: *P. aeruginosa* PAO1 biofilms before LecA-dependent staining.

*P. aeruginosa* PAO1 wt (A, C, E) or the lecA knockout (ΔlecA) mutant (B, D, F) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Z-stacks (232 x 232 µm) were recorded every 2 µm at 561 nm for mCherry (red, C-F upper panels) and 488 nm for fluorescein (green, C-F middle panels). The galleries show only every 6th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm). A and B: 3D images show merged images of both channels (488 nm and 561 nm) from top and side views. Images were background corrected and outliers were removed using imageJ software. C and D: Galleries of background corrected and noise reduced z-stacks. E and F: Galleries of raw z-stacks.
**Figure S24**: LecA-dependent staining of *P. aeruginosa* PAO1 biofilms - raw data

*P. aeruginosa* PAO1 wt (A) or the lecA knockout (ΔlecA) mutant (B) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with the covalent LecA ligand 17 for 10 - 30 min. Z-stacks (232 x 232 µm) were recorded every 2 µm at 561 nm for mCherry (red, A and B, upper panels) and 488 nm for fluorescein (green, A and B middle panels). The galleries show only every 4th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm). Images show raw data.

**Figure S25**: Galleries of LecA-dependent staining of *P. aeruginosa* biofilms with 17. *P. aeruginosa* PAO1 wt (A, identical image to Figure 5A for comparison) or the lecA knockout (ΔlecA) mutant (B, largest identified aggregate of the knockout strain culture) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with the covalent LecA ligand fused to fluorescein (17) for 10 - 30 min. Z-stacks (232 x 232 µm) were recorded every 2 µm at 561 nm for mCherry (red, A and B, upper panels) and 488 nm for fluorescein
(green, A and B middle panels). The galleries show every 4th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm).

**Figure S26**: Three-dimensional imaging of LecA-dependent staining of *P. aeruginosa* PAO1 biofilms with 17. *P. aeruginosa* PAO1 wt (A, identical image to Figure 6A for comparison) or the lecA knockout (ΔlecA) mutant (B, largest identified aggregate of the knockout strain culture) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with 17 for 10 - 30 min. Z-stacks (232 x 232 µm) were recorded every 2 µm at 561 nm for mCherry (red) and 488 nm for fluorescein (green). The 3D images show merged images of both channels (488 nm and 561 nm) from top and side views.
Experimental

General Experimental Details

Commercial chemicals and solvents were used without further purification. D-galactose was purchased from Dextra Laboratories (Reading, UK) and fluorescein isothiocyanate isomer I (FITC) from Sigma-Aldrich (Munich, Germany). Deuterated solvents were from Eurisotop (Saarbrücken, Germany). Thin layer chromatography (TLC) was performed using silica gel 60 coated aluminum sheets containing fluorescence indicator (Macherey & Nagel, Düren, Germany) using UV light (254 nm) and by charring either in aqueous KMnO₄ solution or in a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H₂SO₄) with heating. Medium pressure liquid chromatography (MPLC) was performed on a Teledyne Isco Combiflash Rf200 system using pre-packed silica gel 60 columns from Teledyne Isco, SiliCycle or Macherey-Nagel. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker Avance III 500 UltraShield spectrometer at 500 MHz (¹H) or 126 MHz (¹³C). Chemical shifts are given in ppm and were calibrated on residual solvent peaks. Multiplicities were specified as s (singlet), m (multiplet) or interpreted according to 1ˢᵗ order and higher order where possible. The signals were assigned with the help of ¹H,¹H-COSY, DEPT-135-edited ¹H,¹³C-HSQC and ¹H,¹³C-HMBC experiments. Preparative HPLC-MS was performed on a Thermo Dionex Ultimate 3000 HPLC with UV detection. Analytical HPLC-MS was performed on a Thermo Dionex Ultimate 3000 HPLC coupled to a Bruker amaZon SL for low resolution mass spectra or on a Bruker maxis 4G hr-QqToF spectrometer for high resolution, and the data were analyzed using DataAnalysis (Bruker Daltonics, Bremen, Germany).

1,2:3,4-Di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose (6). 6 was synthesized from 1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose[2] (5, 500 mg, 1.92 mmol) by Swern oxidation following the procedure by Streicher and Wünsch.[3] After chromatography, 6 (348 mg, 1.35 mmol) was obtained as colorless amorphous solid. The NMR of 6 corresponded to the one given in the literature.[4]

6,7-Dideoxy-1,2:3,4-di-O-isopropylidene-α-D-galacto-hept-6-enopyranose (7). 7 was synthesized by treating 6 with a Wittig reagent in analogy to Lehmann and Schäfer.[5] In contrast to the literature, we used methyltriphenylphosphonium iodide and sodium hydride in DMSO to generate
the ylid. Crude olefin 7 was obtained after extraction and was used without purification in the next step. The NMR of the crude product corresponded to literature values.[4]

6,7-Dideoxy-1,2,3,4-tetra-O-acetyl-α/β-D-galacto-hept-6-enopyranose (8). α-8 was first described by Lee et al. and the NMR corresponded to literature values.[4]

Phenyl 2,3,4-tri-O-acetyl-6,7-dideoxy-β-D-galacto-hept-6-enopyranoside (9). 8 (1.24 g, 3.6 mmol) and phenol (678 mg, 7.2 mmol) were dissolved in dry CH2Cl2 (24 mL) and added to a round bottom flask with powdered activated molecular sieves (3Å, 600 mg) under argon. After cooling to 0 °C, BF3•Et2O (1.77 mL, 14.4 mmol) was added in four portions over 1 h. Then, the reaction was stirred for 50 min at 0 °C after which the reaction was allowed to warm to r.t. during 20 min. The reaction was stopped by pouring on cold aqueous satd. NaHCO3, after addition of CH2Cl2 (60 mL) the phases were separated and the organic layer was washed with cold aqueous satd. NaHCO3 (2 x 60 mL). The combined aqueous layers were extracted with CH2Cl2 (3 x 60 mL). The combined organic layers were dried over anhydrous Na2SO4, filtered and the volatiles were removed in vacuo. The residue was purified by MPLC (SiO2, petrol ether/EtOAc gradient of 5-50%) to elute first the anomer α-9 (55 mg, 4%), then the title compound 9 (760 mg, 56%) and then recovered starting material 8 (358 mg, 26%). Analytical data for 9: 1H NMR (400 MHz, CHCl3-d1) δ 7.34 – 7.25 (m, 2H, ArH), 7.10 – 6.99 (m, 3H, ArH), 5.76 (ddd, J = 17.3, 10.7, 4.8 Hz, 1H, H6), 5.51 (dd, J = 10.5, 8.0 Hz, 1H, H2), 5.46 – 5.37 (m, 2H, H4, H7a), 5.27 (dt, J = 10.7, 1.4 Hz, 1H, H7b), 5.15 (dd, J = 10.4, 3.4 Hz, 1H, H3), 5.08 (d, J = 7.9 Hz, 1H, H1), 4.33 (dq, J = 4.7, 1.5 Hz, 1H, H5), 2.14 (s, 3H, CH3CO), 2.06 (s, 3H, CH3CO), 2.01 (s, 3H, CH3CO). 13C NMR (101 MHz, CHCl3-d1) δ 170.56 (CO), 170.30 (CO), 169.56 (CO), 157.30 (ArC), 131.67 (C6), 129.69 (2C, ArCH), 123.30 (ArCH), 118.58 (C7), 117.14 (2C, ArCH), 99.91 (C1), 73.93 (C5), 71.19 (C3), 69.47 (C4), 68.91 (C2), 20.79 (CH3CO), 20.75 (CH3CO). LC-HRMS: [C19H22O8+Na]+ calcd: 401.1207, found: 401.1206.

Phenyl 6,7-dideoxy-β-D-galacto-hept-6-enopyranoside (10). Triacetate 9 (300 mg, 0.79 mmol) was dissolved in dry MeOH (8 mL) under nitrogen. A solution of NaOMe in MeOH (1M, 0.24 mL) was added and the reaction was stirred at r.t. for 2 h. The reaction was neutralized with acidic anion exchange resin (Amberlite IR120/H+), filtered and the volatiles were removed in vacuo. 10 (199 mg, 97%) was obtained as analytically pure colorless amorphous solid. 1H NMR (500 MHz, MeOH-d4) δ 7.31 – 7.23 (m, 2H, ArH), 7.10 – 7.05 (m, 2H, ArH), 7.00 (tt, J = 7.4, 1.1 Hz, 1H, ArH), 5.98 (ddd, J = 17.4, 10.7, 5.5 Hz, 1H, H6), 5.37 (dt, J = 17.3, 1.7 Hz, 1H, H7a), 5.23 (dt, J = 10.7, 1.6 Hz, 1H, H7b), 4.90 (d, J = 7.7 Hz, 1H, H1), 4.20 (dq, J = 5.5, 1.4 Hz, 1H, H5), 3.85 – 3.76
Phenyl 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (6L)-2 and (6D)-3

Olefin 10 (55 mg, 0.22 mmol) was dissolved in dry CH$_2$Cl$_2$ (6 mL) containing 3% MeOH and NaHCO$_3$ (80 mg, 0.95 mmol) was added. After cooling to 0 °C, mCPBA (135 mg, 0.78 mmol) was added portionwise under stirring. Stirring was continued for 10 min and the reaction was allowed to warm to r.t.. After 3 d, mCPBA (135 mg, 0.78 mmol) and NaHCO$_3$ (80 mg, 0.95 mmol) was added. After a reaction time of 10 d, the reaction was diluted with EtOAc (25 mL) and washed with brine (2 x 3 mL). The organic layer was dried over Na$_2$SO$_4$, filtered and the volatiles were removed in vacuo. The aqueous phase contained the desired products and was therefore lyophilized and the combined residues were purified by normal phase flash chromatography to give impure 19 mg of 3 and 17 mg of 2. Both were then separately purified by reversed phase (C18) flash chromatography to give 2 (5.0 mg, 9%) and its epimer 3 (11.0 mg, 19%). The stereochemistry of 2 and 3 was assigned by co-crystallization of the active diastereomer 3 with LecA (vide infra). Analytical data for (6L)-2: 1H NMR (500 MHz, MeOH-d$_4$) δ 7.32 – 7.25 (m, 2H, ArH), 7.13 – 7.07 (m, 2H, ArH), 7.00 (tt, J = 7.5, 1.1 Hz, 1H, ArH), 4.83 (d, J = 7.8 Hz, 1H, H1, signal overlap with residual water), 3.93 (dd, J = 3.4, 1.1 Hz, 1H, H4), 3.82 (dd, J = 9.8, 7.7 Hz, 1H, H2), 3.56 (dd, J = 9.7, 3.4 Hz, 1H, H3), 3.35 – 3.29 (m, 1H, H6, signal overlap with residual solvent), 3.27 (dd, J = 6.7, 1.2 Hz, 1H, H5), 2.83 (dd, J = 4.9, 4.2 Hz, 1H, H7a), 2.71 (dd, J = 5.0, 2.7 Hz, 1H, H7b). 13C NMR (126 MHz, MeOH-d$_4$) δ 159.19 (ArC), 130.38 (2C, ArCH), 123.39 (ArCH), 117.82 (2C, ArCH), 102.81 (C1), 78.79 (C5), 74.49 (C3), 72.06 (C2), 71.48 (C4), 52.89 (C6), 44.42 (C7). LC-HRMS: [C$_{13}$H$_{16}$O$_5$+HCOO]$^-$ calcd: 313.0929, found: 313.0928. Analytical data for (6D)-3: 1H NMR (500 MHz, MeOH-d$_4$) δ 7.32 – 7.22 (m, 2H, ArH), 7.08 – 7.03 (m, 2H, ArH), 7.00 (tt, J = 7.4, 1.1 Hz, 1H, ArH), 4.85 – 4.83 (m, 1H, H1, signal overlap with residual water), 3.98 (dd, J = 3.4, 1.1 Hz, 1H, H4), 3.81 (dd, J = 9.8, 7.7 Hz, 1H, H2), 3.58 (dd, J = 9.8, 3.4 Hz, 1H, H3), 3.44 (dd, J = 5.5, 1.2 Hz, 1H, H5), 3.26 (ddd, J = 5.4, 3.8, 2.7 Hz, 1H, H6), 2.88 – 2.79 (m, 2H, H7a,b). 13C NMR (126 MHz, MeOH-d$_4$) δ 159.11 (ArC), 130.37 (2C, ArCH), 123.39 (ArCH), 117.80 (2C, ArCH), 102.79 (C1), 76.33 (C5), 74.55 (C3), 72.12 (C2), 70.73 (C4), 51.32 (C6), 46.70 (C7). LC-HRMS: [C$_{13}$H$_{16}$O$_2$+HCOO]$^-$ calcd: 313.0929, found: 313.0930.

p-Propargyloxyphenyl 2,3,4-tri-O-acetyl-6,7-dideoxy-β-D-galacto-hept-6-enopyranoside 11. 11 was prepared from 8 (800 mg, 2.32 mmol) and hydroquinone monopropargyl ether[6] (688 mg, 4.64
mmol) as described for 9. Purification of the crude product yielded 11 (627 mg, 62%) as an oil and small amounts of its α-anomer and recovered α-8. Analytical data for 11: 1H NMR (500 MHz, CDCl$_3$-d$_1$) δ 7.03 – 6.95 (m, 2H, ArH), 6.95 – 6.86 (m, 2H, ArH), 5.76 (ddd, $J$ = 17.2, 10.7, 4.8 Hz, 1H, H6), 5.48 (dd, $J$ = 10.5, 8.0 Hz, 1H, H2), 5.45 – 5.39 (m, 2H, H7a, H4), 5.28 (dt, $J$ = 10.8, 1.3 Hz, 1H, H7b), 5.13 (dd, $J$ = 10.5, 3.4 Hz, 1H, H3), 4.97 (d, $J$ = 8.0 Hz, 1H, H1), 4.64 (d, $J$ = 2.4 Hz, 2H, HCCC$_2$-OR), 4.29 (dq, $J$ = 4.6, 1.4 Hz, 1H, H5), 2.51 (t, $J$ = 2.4 Hz, 1H, HCCCH$_2$-OR), 2.15 (s, 3H, CH$_3$CO), 2.08 (s, 3H, CH$_3$CO), 2.01 (s, 3H, CH$_3$CO); 13C NMR (126 MHz, CDCl$_3$-d$_1$) δ 170.59 (CO), 170.33 (CO), 169.58 (CO), 153.65 (ArC), 152.01 (ArC), 131.69 (C6), 118.66 (2C, ArCH), 118.57 (C7), 115.99 (2C, ArCH), 100.84 (C1), 78.72 (HCCCH$_2$-OR), 75.64 (HCCCH$_2$-OR), 73.89 (C5), 71.18 (C3), 69.46 (C4), 68.93 (C2), 56.45 (HCC$_2$H$_2$-OR), 20.92 (C6), 20.82 (CH$_3$CO), 20.77 (CH$_3$CO); LC-HRMS: [C$_{22}$H$_{24}$O$_9$+Na]$^+$ calcd: 455.1313, found: 455.1309.

p-Propargyloxyphenyl 2,3,4-tri-O-acetyl-6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (6L)-12 and (6D)-13.

Oltein 11 (256 mg, 0.59 mmol) was dissolved in CH$_2$Cl$_2$ (12 mL) and cooled to 0 °C. mCPBA (511 mg, 2.96 mmol) was added portionwise under stirring and cooling. Then, the reaction was allowed to warm to r.t. and stirring was continued for 6 d when the reaction was diluted with CH$_2$Cl$_2$ (60 mL) and extracted with satd aq. Na$_2$S$_2$O$_3$, NaHCO$_3$, H$_2$O. The organic layer was dried over Na$_2$SO$_4$, filtered and the volatiles were removed in vacuo. The residue was purified by MPLC (silica, PhMe/EtOAc 5-15%) to give first 13 (119 mg, 46%) and then 12 (55 mg, 21%). Analytical data for (6D)-13: 1H NMR (500 MHz, CDCl$_3$-d$_1$) δ 7.00 – 6.83 (m, 4H, ArH), 5.55 (dd, $J$ = 3.4, 1.0 Hz, 1H, H4), 5.46 (dd, $J$ = 10.5, 7.9 Hz, 1H, H2), 5.09 (dd, $J$ = 10.4, 3.4 Hz, 1H, H3), 4.90 (d, $J$ = 7.9 Hz, 1H, H1), 4.64 (d, $J$ = 2.4 Hz, 2H, HCCCH$_2$-OR), 3.64 (dd, $J$ = 4.7, 1.2 Hz, 1H, H5), 3.16 (ddd, $J$ = 4.7, 3.7, 2.7 Hz, 1H, H6), 2.83 (dd, 1H, $J$ = 5.3, 2.6 Hz, H7a), 2.81 (dd, 1H, $J$ = 5.2, 3.8 Hz, H7b), 2.51 (t, $J$ = 2.4 Hz, 1H, HCCCH$_2$-OR), 2.19 (s, 3H, CH$_3$CO), 2.02 (s, 3H, CH$_3$CO); 13C NMR (126 MHz, CDCl$_3$-d$_1$) δ 170.30 (CO), 170.20 (CO), 169.53 (ArC), 153.65 (ArC), 151.88 (ArC), 118.53 (2C, ArCH), 116.08 (2C, ArCH), 100.83 (C1), 78.72 (HCCCH$_2$-OR), 75.64 (HCCCH$_2$-OR), 73.89 (C5), 71.18 (C3), 69.46 (C4), 68.93 (C2), 56.45 (HCCCH$_2$-OR), 20.92 (CH$_3$CO), 20.82 (CH$_3$CO), 20.77 (CH$_3$CO); LC-HRMS: [C$_{22}$H$_{24}$O$_9$+Na]$^+$ calcd: 449.1442, found: 449.1447. Analytical data for (6L)-12: 1H NMR (500 MHz, CDCl$_3$-d$_1$) δ 7.01 – 6.97 (m, 2H, ArH), 6.94 – 6.89 (m, 2H, ArH), 5.53 – 5.45 (m, 2H, H2+H4), 5.05 (dd, $J$ = 10.4, 3.5 Hz, 1H, H3), 4.92 (d, $J$ = 7.9 Hz, 1H, H1), 4.65 (d, $J$ = 2.4 Hz, 2H, HCCCH$_2$-OR), 3.43 (dd, $J$ = 5.9, 1.2 Hz, 1H, H5), 3.16 (ddd, $J$ = 5.9, 4.2, 2.6 Hz, 1H, H6), 2.80 (dd, $J$ = 4.6, 4.3 Hz, 1H, H7a), 2.66 (dd, $J$ = 4.7, 2.7 Hz, 1H, H7b), 2.51 (t, $J$ = 2.4 Hz, 1H, HCCCH$_2$-OR), 2.21 (s, 3H, CH$_3$CO), 2.08 (s, 3H, CH$_3$CO), 2.02 (s, 3H, CH$_3$CO); 13C NMR (126 MHz, CDCl$_3$-d$_1$) δ 170.53
(CO), 170.27 (CO), 169.50 (CO), 153.73 (ArC), 151.93 (ArC), 118.57 (2C, ArCH), 116.10 (2C, ArCH), 100.76 (C1), 78.72 (HCCCH2-OR), 75.66 (HCCCH2-OR), 75.35 (C5), 70.86 (C3), 68.76 (C2+C4), 56.50 (HCCCH2-OR), 50.89 (C6), 43.57 (C7), 20.89 (2C, CH3CO), 20.74 (CH3CO); LC-HRMS: [C22H25O10]+ calcd: 449.1442, found: 449.1439.

p-Propargyloxyphenyl 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (6L)-14.

Acetylated (6L)-12 (34 mg, 76 µmol) was dissolved in dry MeOH (380 µL) and cooled to 0 °C. Freshly prepared NaOMe (60 mM in MeOH, 570 µL) was added dropwise and the reaction was stirred for 1.5 h at 0 °C. Then, Amberlite IR120/H+ was added, the reaction was filtered and the volatiles were removed in vacuo. Analytically pure (6L)-14 (21 mg, 86%) was obtained.

1H NMR (500 MHz, MeOH-d4) δ 7.06 (d, J = 9.2 Hz, 2H, ArH), 6.91 (d, J = 9.1 Hz, 2H, ArH), 4.72 (d, J = 7.8 Hz, 1H, H1), 4.67 (d, J = 2.4 Hz, 2H, HCCC2-OR), 3.91 (dd, J = 3.5, 1.1 Hz, 1H, H4), 3.79 (dd, J = 9.8, 7.7 Hz, 1H, H3), 3.54 (dd, J = 9.8, 3.4 Hz, 1H, H2), 3.33 – 3.29 (m, 1H, H6), 3.23 (dd, J = 6.7, 1.2 Hz, 1H, H5), 2.91 (t, J = 2.4 Hz, 1H, HCCCH2-OR), 2.83 (dd, J = 4.9, 4.2 Hz, 1H, H7a), 2.70 (dd, J = 4.9, 2.7 Hz, 1H, H7b); 13C NMR (126 MHz, MeOH-d4) δ 154.58 (ArC), 153.83 (ArC), 119.12 (2C, ArCH), 116.86 (2C, ArCH), 103.79 (C1), 79.99 (HCCCH2-OR), 78.74 (C5), 76.54 (HCCCH2-OR), 74.47 (C3), 72.06 (C2), 71.47 (C4), 57.19 (HCCCH2-OR), 52.92 (C6), 44.44 (C7); LC-HRMS: [C16H18O7+HCOO]- calcd: 367.1035, found: 367.1031.

p-Propargyloxyphenyl 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (6D)-15.

Acetylated (6L)-13 (70 mg, 156 µmol) was was treated with NaOMe as described before for its diastereomer. Analytically pure (6D)-15 (47 mg, 94%) was obtained. 1H NMR (500 MHz, MeOH-d4) δ 7.04 – 6.99 (m, 2H, ArH), 6.96 – 6.87 (m, 2H, ArH), 4.72 (d, J = 7.7 Hz, 1H, H1), 4.67 (d, J = 2.5 Hz, 2H, HCCC2-OR), 3.96 (dd, J = 3.4, 1.2 Hz, 1H, H4), 3.78 (dd, J = 9.8, 7.7 Hz, 1H, H2), 3.57 (dd, J = 9.8, 3.5 Hz, 1H, H3), 3.40 (dd, J = 5.4, 1.1 Hz, 1H, H5), 3.26 (ddd, J = 5.4, 3.9, 2.6 Hz, 1H, H6), 2.91 (t, J = 2.4 Hz, 1H, HCCCH2-OR), 2.84 (dd, J = 5.3, 3.9 Hz, 1H, H7a), 2.81 (dd, J = 5.3, 2.7 Hz, 1H, H7b); 13C NMR (126 MHz, MeOH-d4) δ 154.58 (ArC), 153.73 (ArC), 119.14 (2C, ArCH), 116.84 (2C, ArCH), 103.80 (C1), 79.99 (HCCCH2-OR), 76.55 (C5), 76.26 (HCCCH2-OR), 74.52 (C3), 72.15 (C2), 70.71 (C4), 57.18 (HCCCH2-OR), 52.92 (C6), 44.44 (C7); LC-HRMS: [C16H18O7+HCOO]- calcd: 367.1035, found: 367.1031.

Fluoresceine coupled 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (6D)-17.

Propargylated epoxide (6D)-15 (1.28 mg, 4 µmol) and azide modified FITC 16[7] (2.8 mg, 4 µmol) were dissolved in DMF (120 µL). After the addition of CuSO4*5H2O (0.3 mg, 1.2 µmol) and sodium ascorbate (80 µL, 100 mM in H2O, 8 µmol), the reaction was stirred at r.t. for 3 h. After
lyophilization, the residue was purified by preparative HPLC (C18, H2O/MeCN, gradient of 20-60%) to give pure (6D)-17 (2.8 mg, 76%). LC-MS: [C45H47N5O15S+H]+ calcd: 930.3, found: 930.3.

**Competitive binding to LecA using fluorescence polarization**

The protein LecA was expressed and purified as described previously.[8,9] The competitive binding assay was performed as described previously.[9] In brief, to 20 µL of a stock solution of LecA and fluorescent ligand (final assay concentration 10 nM) in TBS/Ca were added 10 µL serial dilutions of test compounds in TBS/Ca in triplicates in black 384-well microtiter plates (Greiner Bio-One, Germany, cat no 781900). After addition of the reagents, the microtiter plates were centrifuged at 800 rpm for 1 min at 23 °C and subsequently incubated for 4-6 h at r.t. in a humidity chamber on a rocking table. Fluorescence was measured on a PheraStar FS plate reader (BMG Labtech GmbH, Germany) with excitation filters at 485 nm and parallel and perpendicular emission filters at 535 nm. The measured intensities were reduced by the values of only LecA in buffer. The fluorescence polarization data were calculated and analyzed with MARS Data Analysis Software (BMG Labtech GmbH, Germany) and fitted according to the four parameter variable slope model. Bottom and top plateaus were defined by the standard compounds included as controls in each assay (methyl α-D-galactoside and phenyl β-D-galactoside, respectively) and the data was reanalyzed with these values fixed. A minimum of three independent measurements on three plates was performed for each inhibitor. For binding kinetics, fluorescence polarization was measured over a period of 3 days at r.t. or 37 °C.

**Complex formation of LecA with covalent fluorescent reporter ligand 17 and the non covalent control 18 and displacement using a competitive inhibitor**

A stock solution of LecA (600 µM) was incubated with 17 (100 µM) or with the previously described galactose-based ligand 18[9] (100 µM) in TBS/Ca (20 mM Tris, 137 mM NaCl, 2.6 mM KCl at pH 7.4 supplemented with 1 mM CaCl2 and 2.5% DMSO) at 25 °C for 48 h (Figure S22). The resulting complexes were distributed in a black 384-well microtiter plate, 20 µL per well and mixed with with 10 µL of serial dilutions (150 mM to 48 µM) of methyl α-D-galactoside in TBS/Ca in duplicates. After incubation for 1 h at r.t., fluorescence polarization was determined as described above. Fluorescence polarization was normalized where values of unbound ligand 17 or 18, respectively, were set to 0% effect and those wells with the lowest methyl α-D-galactoside content were set to 100% effect. The data was fitted according to the four parameter variable slope model.
LecA and the complex of LecA with 17 was analyzed after heat denaturation in Lämmli buffer (5 min, 95 °C) by 17% SDS-PAGE. Gels were analyzed by fluorescence scanning on a Typhoon 9400 device (GE Healthcare) at 488 nm and subsequent Coomassie staining.

**Mass spectrometry**

Analyzed LecA samples with inhibitors 1 or 3 were taken from the competitive binding assay containing 3 mM (intact protein) or 1 mM (MALDI) inhibitor after 24 h incubation. All intact protein ESI-MS-measurements were performed on a Dionex Ultimate 3000 RS/LC system using an Aeris Widepore XB-C8, 150 x 2.1 mm, 3.6 μm dp column (Phenomenex, USA). Separation of 0.5 μL sample was achieved by a linear gradient from (A) H₂O + 0.1% HCOOH to (B) MeCN + 0.1% HCOOH at a flow rate of 300 μL/min and 45 °C. The gradient was initiated by a 1.0 min isocratic step at 2% B, followed by an increase to 75% B in 10 min to end with a 3 min step at 75% B before reequilibration with initial conditions. UV spectra were recorded by a DAD in the range from 200 to 600 nm. The LC flow was split to 75 μL/min before entering the maXis 4G hr-ToF mass spectrometer (Bruker Daltonics, Bremen, Germany) using the standard Bruker ESI source. In the source region, the temperature was set to 180 °C, the capillary voltage was 4000 V, the dry-gas flow was 6.0 L/min and the nebulizer was set to 1.1 bar. Mass spectra were acquired in positive ionization mode ranging from 600-1800 m/z at 2.5 Hz scan rate. Protein masses were deconvoluted by using the Maximum Entropy algorithm (Spectrum Square Associates, Inc.).

All MALDI-ToF measurements were acquired on a Bruker ultraflXtreme MALDI-ToF/ToF mass spectrometer (Bruker Daltonics, Germany) equipped with a smartbeam II solid state 1 kHz laser. In-source decay (ISD) experiments for top-down sequencing were performed in positive ion reflectron mode ranging from 4000-9000 m/z. The acceleration voltage was set to 25.00 kV, extraction voltage to 22.55 kV, lens voltage was held at 7.5 kV and reflector voltage was set to 26.45 kV. The ISD spectra comprised 80000 accumulated laser shots (arbitrary laser power of 20%) and were externally calibrated using c-type fragment ions generated from intact ubiquitin. All ISD samples were prepared by pre-mixing sample solution (250 μM protein in H₂O) and matrix solution (1,5-diaminonaphthalene saturated in MeCN - H₂O + 0.1% TFA (50:50)) in a ratio of 1:2 (v:v) and spotted 0.5 μL onto a ground steel target.

**Crystallization and structure determination**

LecA dissolved in water to 10 mg/mL was co-crystallized with 2 mM ligand 3 after incubation during one day at room temperature. Crystallization screening was performed using the vapor
diffusion method. Sitting drops of 200 nl drops containing a 1/1 (v/v) mix of protein and reservoir solution at 20 °C were made using the robot of the HTXlab, Grenoble, France. Crystal clusters were obtained in five days from solution 6 from the PEGs-I screen (Qiagen): 25% peg 2KMME and 0.1M sodium acetate pH 4.6. A broken part of the cluster was directly mounted in a cryoloop and flash-freezed in liquid nitrogen. Diffraction data were collected at 100 K at the European Synchrotron Radiation Facility (Grenoble, France) on BM30A-FIP using an ADSC Q315r detector. The data were processed using XDS. All further computing was performed using the CCP4 suite. Data quality statistics are summarized in Table S1. The structure was solved by molecular replacement using PHASER and the tetramer coordinates of PDB-ID 1OKO as search model. This model was initially rebuild using ARP/WARP and then the structure was refined with restrained maximum likelihood refinement using REFMAC 5.8 and local NCS restrains iterated with manual rebuilding in Coot. Five percent of the observations were set aside for cross-validation analysis, and hydrogen atoms were added in their riding positions and used for geometry and structure-factor calculations. Incorporation of the ligand was performed after inspection of the ARP/WARP 2Fo-DFc weighted maps. Water molecules, introduced first with ARP/WARP and then automatically using Coot, were inspected manually. The model was validated with the wwPDB Validation server: http://wwpdb-validation.wwpdb.org. The coordinates were deposited in the Protein Data Bank under code 5MIH.

Microbiology

Generation of fluorescent P. aeruginosa strains

Bacterial cultures were grown in LB medium at 37 °C and 180 rpm. P. aeruginosa PAO1 wt (DSM19880) and P. aeruginosa PAO1 ΔlecA (source Bodo Philipp, University of Münster) were transformed with mCherry expressing plasmid pMP7605 by three parental mating using E. coli DH5α (source Rolf Müller lab) as donor strain and E. coli HB101 [RK 600] (source Bodo Philipp, University of Münster) as helper strain. Bacterial pre-cultures were inoculated from single colonies in 5 mL LB with appropriate antibiotics (30 µg/mL chloramphenicol for bacteria containing pRK600; 15 µg/mL gentamicin for for bacteria containing pMP7605) and grown at 37 °C and 180 rpm overnight to stationary phase. For each mating, 100 µL pre-culture was combined and washed twice with pre-warmed LB medium. Strain mixtures were resuspended in 30 µL pre-warmed LB medium and spotted onto pre-warmed LB agarose plates and incubated overnight at 37 °C. After 24 hours from each plate all growth was resuspended in 1 mL LB per plate. 100 µL of the bacterial suspension and 100 µL of a 1:10 dilution were plated on LB-gentamicin (120 µg/mL) agar plates.
and incubated at 37 °C for 24 hours. Successfully transformed bacteria were gentamicin-resistant and the colonies were identified by their pink color and restreaked.

**Biofilm experiments**

Bacterial pre-cultures of *P. aeruginosa* PAO1 w.t. pMP7605 or ΔlecA pMP7605 were inoculated from single colonies in 5 mL LB and grown at 37 °C and 180 rpm overnight to stationary phase. For the biofilm assay the bacterial pre-cultures were diluted to an OD600nm of 0.02 in fresh LB medium. 400 µL bacterial culture were transferred to each well of a 24-well imaging plate (cat no 3231, zell-kontakt GmbH, Germany). Plates were incubated at 37 °C and shaking at 180 rpm for 20 - 24 hours.

FITC-labelled epoxide 17 was suspended in H₂O/10% DMSO (0.84 mg/mL), centrifuged and the supernatant was used in the staining experiments. Biofilms were stained for the presence of LecA by the addition of 44 µL FITC-labelled epoxide 17 to each well. After an incubation time of 10 - 30 min at r.t., bacterial biofilms were directly visualized by measuring the fluorescence of the fluorescein-labeled LecA ligand and mCherry using a confocal laser scanning microscope (Leica TCS Sp8 CLSM). Fluorescein was excited with a 488-nm laser and mCherry at 561 nm. Focal planes were acquired starting from the bottom of the plate (position 0) with an interplane distance (z-step size) of 2 µm using a 25× numerical-aperture water objective. Images were batch-processed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016.) for background correction and noise filter ("remove outliers").

**Supporting Information References**


